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(54) Stabilized compositons of reverse transcriptase and RNA polymerase for nucleic acid amplification

Stabilisierte Zusammensetzungen von Reverser Transkriptase und RNA Polymerase für Nukleinsäureamplifikation

Compositions stabilisées contenant les enzymes transcriptase reverse et ARN polymérase pour l'amplification d'acides nucléiques

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Description

Field of the Invention

[0001] This invention relates to the fields of molecular biology, nucleic acid amplification and stabilized biological compositions generally. In particular, the present invention relates to a stable lyophilized enzyme composition containing one or more nucleic acid polymerases.

Background of the Invention

[0002] Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are large linear macromolecules composed of covalently-linked nucleotide subunits. DNA is usually found in a "double-stranded" form in which two DNA chains are associated by hydrogen bonding in an antiparallel fashion. RNA usually exists in nature as a single polynucleotide chain. Nucleotides are molecules having a sugar (either deoxyribose or ribose) and a nitrogenous base moiety, and are usually connected together in nucleic acids by a phosphodiester linkage. There are five common nitrogenous bases. Three are found in both DNA and RNA: these are adenine (A), guanine (G) and cytosine (C). The other two, thymine (T) and uracil (U), are specific to DNA and RNA, respectively.

[0003] Most (if not all) of every organism's genetic information is transmitted from one generation to the next in the form of DNA or RNA. This information is conveyed in the sequence of the nucleotides along a single nucleic acid chain or "strand", which constitutes a genetic code. Moreover, each of the nitrogenous bases of a nucleic acid strand has the ability to specifically hydrogen bond with one or more other nitrogenous bases of the same or a different nucleic acid strand. Thus, under usual conditions, A hydrogen bonds with T (or U), and C hydrogen bonds with G; this specific hydrogen-bonding is called base-pairing. In double-stranded DNA each of the two strands consists of a chain of nucleotides in which most or all of the nucleotides are base-paired with nucleotides of the other strand. In such a case, the order of nucleotides on one DNA strand determines the order of nucleotides on the other DNA strand. Two nucleic acid strands which are "mirror images" of each other in this way are said to be perfectly complementary.

[0004] Nucleic acids are synthesized in vivo by a mechanism exploiting the fact that each nucleic acid strand dictates the order of nucleotides of a perfectly complementary strand; this remains true whether the desired nucleic acid is RNA or DNA, and regardless whether the nucleic acid to be used as a template is RNA or DNA. Most of the specific mechanisms for DNA replication involve the use of a DNA polymerase to sequentially add nucleotides to a 3' hydroxyl group of a polynucleotide primer hydrogen-bonded to the template nucleic acid strand. The newly added nucleotides are chosen by the DNA polymerase based on their ability to base-pair with the corresponding nucleotide of the template strand. This process of adding nucleotides to one end of a primer is sometimes called primer extension.

[0005] Unlike DNA synthesis, RNA synthesis does not normally require the existence of a polynucleotide primer. Rather, RNA synthesis is usually mediated by an RNA polymerase which recognizes one or more specific nucleotide sequences of a nucleic acid template. The region of the template to which the RNA polymerase binds, called a promoter, is usually double-stranded. After binding to the promoter, the RNA polymerase "reads" the template strand and synthesizes a covalently-linked polyribonucleotide strand complementary to the template. RNA polymerases from different organisms preferentially recognize different promoter sequences.

[0006] DNA and RNA polymerase enzymes have been purified from a number of diverse organisms. Some of these enzymes, such as E. coli DNA polymerase I, the Klenow fragment of DNA polymerase I, and various RNA polymerases are commonly used in vitro as tools in molecular biology and nucleic acid biochemistry research. See generally e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed. Cold Spring Harbor Press 1989).

[0007] Another use for nucleic acid polymerases has arisen with the advent of various methods of nucleic acid amplification, such as the polymerase chain reaction (PCR), see e.g., Mullis et al., U.S. Patents Nos. 4,683,195, 4,683,202, and 4,800,159. In the simplest form of PCR, two oligonucleotide primers are synthesized, each primer complementary to a region of a target nucleic acid positioned to the 3'side, with respect to the target nucleic acid, of a target nucleotide sequence region. Each primer is complementary to one of two complementary nucleic acid strands; the target region comprises a nucleotide sequence region encompassing both nucleic acid strands of a double-stranded target nucleic acid. When these primers are allowed to hydrogen-bond ("hybridize") with the substrate and a DNA polymerase is added to the reaction mixture along with nucleotide triphosphates, each hybridized primer is extended by the enzyme in a 5'→ 3' direction. The reaction mixture is then heated to melt the primer extension product:template hybrid, the temperature is decreased to permit another round of primer/target hybridization, and more DNA polymerase is added to replace the DNA polymerase inactivated by the high temperature step. By repeating the process through a desired number of cycles, the amount of nucleic acids having the target nucleotide sequence is exponentially increased. More recently, a thermostable DNA polymerase derived from Thermus aquaticus has been successfully used in the PCR method to lessen the need for repeated addition of large amounts of expensive enzyme. The Taq polymerase resists inactivation at 90-95°C, thus obviating the need for repeated additions of enzyme after each round of strand separation.

[0008] Other methods of nucleic acid amplification have been devised, such as those using RNA transcription as a step in the amplification process. One such method functions by incorporating a promoter sequence into one of the primers used in the PCR reaction and then, after amplification by the PCR method, using the double-stranded DNA as a template for the transcription of single-stranded RNA by a DNA-directed RNA polymerase, see e.g., Murakawa et al., DNA 7:287-295 (1988)).

[0009] Other amplification methods use multiple cycles of RNA-directed DNA synthesis and transcription to amplify DNA or RNA targets, see, e.g., Burg et al., WO 89/1050; Gingeras et al., WO 88/10315 (sometimes called transcription amplification system or TAS); Kacian and Fultz, EPO Publication No. EPO 408,295 (which enjoys common ownership with the present application); Davey and Malek, EPO Application No. 88113948.9; Malek et al., WO91/02818). These methods make use of an enzyme, reverse transcriptase (RT), which can use RNA or DNA as a template for synthesis of a complementary DNA strand. Some of these methods also utilize cellular RNAse H activity as an essential component. Most retroviral reverse transcriptases, such as those encoded by Moloney Murine Leukemia Virus (MMLV) and Avian Myeloblastosis Virus (AMV), possess an RNA-directed DNA polymerase, a DNA-directed DNA polymerase activity as well as RNaseH activity. RNAse H activity selectively degrades the RNA strand of an RNA:DNA hybrid nucleic acid molecule, thus allowing the amplification reaction to proceed without the need for temperature cycling.

[0010] Nucleic acid amplification is an increasingly popular tool for the specific identification and/or amplification of unique or characteristic nucleic acid segments in a variety of settings. Thus, nucleic acid amplification is used in food and agricultural testing, medical diagnostics, human genetic testing and counseling, archeology, and criminal forensics. Because these methods all utilize enzymes, methods of producing, packaging, transporting and storing large quantities of highly active enzymes has become an issue of critical importance in the manufacture, marketing and sale of enzymes and kits for nucleic acid amplification. Specifically, for methods employing transcription-based amplification, commercially acceptable methods and preparations for storing active preparations of reverse transcriptase and RNA polymerase are necessary for the successful manufacture and marketing of kits for nucleic acid amplification.

[0011] The usual method of stabilizing reverse transcriptase and RNA polymerase enzymes (as well as many other enzymes used in molecular biology research) is by storing a liquid preparation of each enzyme in a solution containing 50% (v/v) glycerol and a reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol (βME) at -20°C. This method preserves the activity of the enzymes for many months with little loss of activity. By contrast, enzyme activities are readily lost when the enzymes are stored at room temperature or at 4°C. These preparations are generally shipped from the enzyme supplier to the end user in dry ice; losses of 30% or more of enzyme activity are common during such transport due to freezing and thawing of the enzyme preparation. These enzymes are formulated and supplied separately.

[0012] A method of storing and shipping reverse transcriptase and RNA polymerase without the need for refrigeration would obviate the necessity for refrigerated transport and/or methods of cold storage such as dry ice, wet packs, dry packs, or styrofoam shipping containers. Such methods would also be more cost effective, since the production overhead associated with these methods of maintaining enzyme activity would be unnecessary. Methods of storing enzymes which would allow the enzyme preparation to tolerate a limited exposure to higher temperatures would eliminate the losses in enzyme activity which could result if the enzyme preparation sits on a loading dock or in a truck during shipment. Such a method would have to be highly reproducible. Moreover, if the enzymes could be provided in a single container in a form compatible with their intended use (such as in a formulation containing all or most of any necessary co-factors and substrates) such a preparation would be more economical to manufacture and more convenient to use.

[0013] Freeze-drying (lyophilization) has been used to preserve foods, biological membranes, whole cells (see, e.g., American Society for Microbiology, Manual of Methods for General Bacteriology 210-217 (1981), and biological macromolecules including enzymes. Lyophilization involves the removal of water from a frozen sample by sublimation under lowered pressure. Sublimation is the process by which a solid is evaporated without passing through the liquid stage.

[0014] The theoretical aspects of lyophilization are complex. It is thought that when a biological substance such as a protein is in aqueous solution the molecule is surrounded by a hydration shell comprising water molecules; this hydration shell stabilizes the protein and helps maintain its activity. When water is removed, the protein's reactive groups, which are normally masked by the hydration shell, are free to react with each other, thus forming new, essentially irreversible bonds. These bonds can distort the protein's native conformation. Also, new hydrophobic/hydrophilic interactions may take place in the absence of water which also can distort the conformation of the protein. Since the three-dimensional conformation of many proteins confers a biological activity, the distortion of the conformation can alter biological activities upon drying. By the same mechanism, cross-linking and aggregation of proteins can occur.

[0015] Freezing the protein sample prior to drying helps reduce the degree of conformational distortion due to drying. The lowered initial temperature helps keep unwanted reactions between amino acid reactive groups to a minimum by depriving the reactants of energy. At the same time, while in a frozen state the protein has less stearic freedom than when in solution and is less prone to gross conformational change.

[0016] However, completely dried lyophilizates tend to have a shorter "shelf" or storage life than do incompletely

dried lyophilizates still containing a low percentage of water. Such incompletely dried lyophilizates must often be stored at temperatures no higher than about 4-10°C, and are still capable of undergoing inactivating chemical reactions that would not be possible were water not present. Thus, while the shelf life of many incompletely dried lyophilized biologically active proteins is longer than those that are completely dried, it is still necessary to refrigerate the preparation in order to maintain activity. Even so, there is a loss of activity in such preparations over a relatively short period of time. Moreover, some enzymes, such as phosphofructokinase, are completely inactivated after lyophilization in the absence of a cryoprotectant, regardless of whether the preparation is completely dried or not. See e.g., Carpenter et al., Cryobiology 25:372-376 (1988).

[0017] As used herein, the term "cryoprotectant" is intended to mean a compound or composition which tends to protect the activity of a biologically active substance during freezing, drying, and/or reconstitution of the dried substance.

[0018] The term "stabilizing agent" is meant to mean an agent that, when added to a biologically active material, will prevent or delay the loss of the material's biological activity over time as compared to when the material is stored in the absence of the stabilizing agent.

[0019] A variety of cryoprotectant additives have been used or proposed for use as excipients to help preserve biological activity when biological materials, including particular proteins, are dried. Clegg et al., Cryobiology 19: 106-316 (1982) have studied the role of glycerol and/or trehalose in the ability of cysts of the brine shrimp Artemia to remain viable after desiccation. Carpenter et al., Cryobiology 24: 455-464 (1987), report that the disaccharides maltose, sucrose, lactose and trehalose can play a role in increasing the stabilization of phosphofructokinase activity in a purified enzyme preparation subjected to air-drying. EPO Publication No. 0431882A2, discloses a stabilized preparation of purified alkaline phosphatase that had been derivatized and then lyophilized in the presence of mannitol or lactose. EPO Publication No. 0091258A2, discloses a method for stabilizing tumor necrosis factor (TNF) by storage or lyophilization of the purified protein in the presence of a stabilizing protein, such as human serum albumin, gelatin, human γ globulin, or salmon protamine sulfate. U.S. Patent No. 4,451,569 discloses the use of pentoses, sugar alcohols and some disaccharides to stabilize the activity of purified glutathione peroxidase. The stabilized composition may be freeze-dried and then stored at temperatures below 20°C. EPO Publication No. 0448146A1 discusses stabilized, lyophilized gonadotropin preparations containing a dicarboxylic acid salt. The preparation can further contain a disaccharide such as sucrose or trehalose. Roser, Biopharm, 47-53 (September 1991) discusses preserving the biological activity of various biological molecules dried at ambient temperature using trehalose. PCT Publication No. WO87/00196 reports the stabilization of monoclonal antibodies and calf intestine alkaline phosphatase by air drying in the presence of trehalose. PCT Publications WO89/00012 and WO89/06542 discuss the use of trehalose to preserve some foods and the antigenicity of live virus particles. EPO Publication 02270799A1 reports the stabilization of recombinant β-interferon in a formulation containing a stabilizing agent such as a detergent or glycerol. The compositions can further comprise various sugars including sucrose and trehalose, sugar alcohols, and proteins as additional stabilizing agents; most preferred among these is dextrose.

[0020] Some of these additives have been found to extend the shelf life of a biologically active material to many months or more when stored at ambient temperature in an essentially dehydrated form. However, the effectiveness, suitability or superiority of a particular prospective additive depends on the chemical composition of the biologically active material sought to be stabilized; in the case of a protein these factors may include, without limitation, the amino acid sequence of the protein, and its secondary, tertiary and quaternary structure. Thus, whether a particular composition will function to preserve biological activity for a particular biologically active material is not a priori predictable.

[0021] Moreover, if a protein is lyophilized, additional factors including, without limitation: the buffer composition, the speed of freezing, the amount of negative pressure, the initial, operating and final lyophilization temperatures and the length of the lyophilization procedure are important in determining the stability and shelf life of the active protein.

[0022] Some proteins are known to have multiple enzymatic activities. Thus, retroviral reverse transcriptase enzymes such as those derived from Moloney Murine Leukemia Virus (MMLV-RT) have a DNA-directed DNA polymerase activity, an RNA-directed DNA polymerase activity, and an RNAse H activity. While these activities are contained in the same enzyme, conditions for the preservation of any one of these activities in a dried preparation does not assure that one or both of the remaining enzyme activities will also be preserved under the same conditions.

[0023] Moreover, when a particular application requires that the balance of relative specific activities of the three activities of reverse transcriptase remain similar after reconstitution to the balance of these activities before drying, as in the transcription-based nucleic acid amplification system of Kacian & Fultz, supra (which enjoys common ownership with the present application and is incorporated by reference herein), a particular preservation method may upset the delicate balance of these enzymatic activities, thereby making the enzyme unsuitable for such use. Thus, if the RNaseH activity of the enzyme is preserved more than the RNA-directed DNA polymerase activity, the RNA:DNA initiation complex may be degraded before DNA synthesis can begin.

[0024] Since a given cryoprotectant composition effective for the long-term preservation of a given enzymatic activity is not clearly effective or superior when applied to another enzymatic activity, diff rent enzymes often require quite different protestants for activity stabilization. As a result, among commercially manufactured lyophilized enzyme prep-

arations, all or most contain only a single enzyme dried in a formulation customized to preserve the activity of that specific enzyme.
WO-A-93 00807 discloses a method for stabilizing biomaterials during lyophilization including a solution containing a sugar and polyvinylpyrrolidone as stabilizing agents and RNA polymerase / reverse transcriptase as individual proteins to be part of the composition to be freeze-dried. However, this prior art teaching does not provide any suggestion to co-lyophilize a mixture of RMA polymerase and reverse transcriptase, or any other set of proteins.

Summary of the Invention

[0025] The present invention is directed to compositions and kits comprising dried formulations of reverse transcriptase and RNA polymerase able to be stored at ambient temperature for prolonged periods of time without substantial losses of enzymatic activities. Preferably, the formulations comprise preparations of retroviral reverse transcriptase and/or bacteriophage RNA polymerase.

[0026] More preferably, the formulations comprise reverse transcriptase derived from Moloney Murine Leukemia Virus (MMLV-RT) and bacteriophage T7 RNA polymerase in a cryoprotectant excipient. Even more preferably, the invention is directed to single containers comprising dried formulations containing both MMLV-RT and T7 RNA polymerase in one or more cryoprotectant excipients. Most preferably, the invention is directed to single containers comprising dried formulations containing MMLV-RT and T7 RNA polymerase, one or more cryoprotectant excipients comprising either or both trehalose and polyvinylpyrrolidone (PAP), nucleotide triphosphates, and metal ions and co-factors necessary for said enzymatic activities wherein, upon reconstitution of the stabilized lyophilizate and addition of a target nucleic acid and one or more appropriate primers, the formulation is in a convenient and cost-effective form for nucleic acid amplification without the need for excessive handling. Optionally, such a formulation may contain primers for initiation of nucleic acid synthesis. Lastly, the present invention is directed to methods of making and using the dried formulations described above.

[0027] Reverse transcriptase and RNA polymerase enzymes are important agents in transcription-mediated nucleic acid amplification methods, such as those described in Burg et al., supra; Gingeras et al., supra, (sometimes called transcription amplification system or TAS); Kacian and Fultz, supra; Davey and Malek, EPO Application No. 88113948.9 and Malek et al., PCT Publication No. WO91/02818). Such methods are increasingly important in fields such as forensics and medical diagnostics, where the stability of the amplification reagents over time is a significant consideration in the cost of manufacturing, marketing and use of products which employ nucleic acid amplification.

[0028] Applicant has discovered a method and a dried formulation for the preservation of the DNA-directed DNA polymerase, RNA-directed DNA polymerase, and RNAse H activities of reverse transcriptase. The same method and formulation has been discovered to be suitable for the preservation of RNA polymerase activity. Moreover, Applicant has surprisingly found that both enzymes and all four enzymatic activities can be stabilized and preserved as a dried formulation in a single container without significant loss of any of the four activities over a substantial period of time, even after prolonged incubation at high temperature.

[0029] One aspect of the present method comprises providing an active purified reverse transcriptase with a cryoprotectant excipient comprising a non-reducing disaccharide (preferably sucrose or trehalose), or polyvinylpyrrolidone (PAP), or an amount of a mixture of these compounds effective to act as an agent protecting and preserving the DNA-directed DNA polymerase, RNA-directed DNA polymerase, and RNAse H activities of reverse transcriptase after drying the enzyme by methods such as, without limitation, lyophilization of a solution containing reverse transcriptase and the cryoprotectant.

[0030] In a second aspect, the invention features a method for stabilizing and preserving active purified RNA polymerase, preferably T7 RNA polymerase, in a dehydrated form substantially stable at room temperature for more than 90 days. In this aspect, the RNA polymerase is dried in the presence of metal salts, such as those containing Mg^{++} or Zn^{++}, one or more protective stabilizing agents selected from the group consisting of non-reducing disaccharides, preferably trehalose, and polyvinylpyrrolidone (PAP), and a reducing agent, such as n-acetyl-L-cysteine (NALC). While not wishing to be limited by theory, Applicant believes that the reducing agent helps to prevent inactivation of the enzyme through oxidation of any cysteine residues present in the enzyme. In this aspect, the RNA polymerase retains at least 70% of its original activity, preferably after exposure of the dehydrated formulation to a temperature of 45°C for at least 30 days or 35°C for at least 61 days.

[0031] In another aspect, the invention features a single dried formulation containing a mixture of reverse transcriptase (preferably MMLV-RT), RNA polymerase (preferably T7 RNA polymerase), an amount of a cryoprotectant excipient (preferably trehalose and/or polyvinylpyrrolidone) effective to preserve the enzymatic activities of the dried enzymes, nucleotide triphosphates, necessary co-factors, optional oligonucleotide primers, and a reducing agent, preferably a thiol compound.

[0032] In yet another aspect, the present invention comprises a component of a kit for the amplification and specific identification of nucleic acids belonging to one or more phylogenetic groupings of organisms, for example for the specific

detection of one or more species within a genus or one or more genera within a family. The invention provides a reconstitutable dried formulation comprising a reverse transcriptase, an RNA polymerase, ribonucleotide triphosphates, deoxyribonucleotide triphosphates, zinc and/or magnesium salts, and a reducing agent in a single container. Amplification primers and an aqueous reconstitution solution may be supplied as one or more additional separate components of the kit. Alternatively, amplification primers may be comprised in the dried formulation. Target sequence-specific nucleic acid hybridization assay probes and any desired unlabeled helper oligonucleotides may be included in the dried formulation or provided in a separate reagent. Upon reconstitution of the dried formulation and addition of the oligonucleotide primers (if not already present), the mixture is contacted with a partially or wholly single-stranded target nucleic acid. If the target nucleic acid has nucleotide sequences complementary to the primer(s) (or the primer portion of a promoter-primer(s)), the reaction will proceed upon incubation of the reaction mixture at a temperature sufficient for nucleic acid amplification.

[0033] In another aspect, the invention comprises a single lyophilizate containing a combination of reverse transcriptase (preferably MMLV-RT), RNA polymerase (preferably T7 RNA polymerase), a cryoprotectant excipient, nucleotide triphosphates, necessary co-factors and a reducing agent, preferably containing a thiol group. The lyophilizate may be transported and stored without the need for refrigeration, and can withstand transient exposure to elevated temperatures, for example, without limitation, 55°C for 30 days, without significant diminution of enzyme activity.

[0034] By "nucleotide triphosphates" is meant ribo- or deoxyribonucleotide triphosphates and derivatives thereof which are able to serve as substrates for an RNA polymerase and a DNA polymerase, preferably a reverse transcriptase, respectively. Such derivatives may include, without limitation, nucleotides having methyl (or other alkyl) and/or sulfur groups incorporated at the nitrogenous base (usually adenine, thymine or uracil, cytosine and guanine), the ribose or deoxyribose moiety, or the phosphate group.

[0035] By "nucleotide" is meant a nucleic acid subunit comprising a single nitrogenous base (usually adenine, thymine or uracil, cytosine and guanine), a sugar moiety (ribose or deoxyribose) and a phosphate group. As used herein, the term refers both to unincorporated ribo- or deoxyribonucleotide triphosphates and to the covalently-linked nucleotide subunits of an oligonucleotide or nucleic acid strand, depending upon the context of usage.

Detailed Description of the Invention

[0036] The present invention involves methods for stabilizing the enzymatic activities of DNA polymerase and RNA polymerase enzymes by removing the solvent from a solution containing one or more of these enzymes in the presence of a cryoprotectant, or stabilizing "bulking agent". Such cryoprotectants include saccharides, particularly non-reducing disaccharides, and water soluble polymers having electropositive and/or electronegative groups available for hydrogen-bonding with the enzyme. Particularly preferred cryoprotectants are the disaccharides sucrose and trehalose and the polymer polyvinylpyrrolidone (PAP).

[0037] The present invention also relates to stabilized compositions comprising a desiccated DNA polymerase, a desiccated RNA polymerase, or a desiccated mixture containing both a DNA polymerase and an RNA polymerase. Preferred enzymes comprising these compositions are reverse transcriptases and bacteriophage RNA polymerases; particularly preferred enzymes are the retroviral reverse transcriptase from Moloney Murine Leukemia Virus and the RNA polymerase from bacteriophage T7.

[0038] A preferred method of desiccating the DNA polymerase and RNA polymerase of the present invention is by lyophilization. In this process, a solution containing the enzyme is frozen, a vacuum applied to the frozen enzyme solution, and the solvent removed from the preparation by sublimation, leaving behind the solutes.

[0039] The present invention also features a composition for the replication of one or more particular nucleic acid sequences which includes a desiccated preparation of a DNA polymerase (preferably a reverse transcriptase), an RNA polymerase, nucleotide triphosphates, and co-factors necessary for enzyme activity. The desiccated preparation may also contain amplification primers for the specific replication of the target nucleotide sequence and/or hybridization assay probes and helpers. Preferably, the desiccated composition is prepared by lyophilization.

[0040] The compositions of the present invention are stable for a prolonged period, even when stored at high temperatures. Such compositions are thus useful in shipping and storage of commercial preparations of these enzymes and of kits for nucleic acid amplification which contain these enzymes.

Examples

[0041] It will be understood that the following examples are intended to illustrate various presently preferred embodiments of the present invention and do not in any way limit its scope. Nor is the disclosure of an embodiment a representation that other embodiments of the invention might not exist which are more effective to achiev one or more object sought to be addressed by the present invention.

[0050] The amount of amplified nucleic acid produced during the reaction was determined using the homogeneous protection assay described in Arnold and Nelson, U.S. Patent No., 5,283,174 (which enjoys common ownership with the present application and which is incorporated by reference herein); it will be clear to one of skill in the art that many other assay systems and methods of detecting a nucleic acid target, such as by employing radiolabeled probes, are available in the art.

[0051] The amplification reaction was terminated with the addition to each tube of 100 µl of a hybridization buffer containing 200 mM lithium succinate (pH 5.2), 17% (w/v) lithium lauryl sulfate, 3 mM EDTA (ethylenediamine tetraacetic acid) and 3 mM EGTA ([ethylenebis (oxyethylenitrilo)]-tetraacetic acid)) and an acridinium ester-labeled probe (SEQ ID NO: 3) complementary to the T7 Gene 10 RNA transcript. The tubes were incubated at 60°C for 20 minutes. The acridinium ester associated with unhybridized probe was hydrolyzed with the addition of 300 µl of 182 mM NaOH, 600 mM boric acid and 1% (v/v) TRITON® X-100 and the tubes incubated at 60°C for 5 minutes. The remaining chemiluminescence was measured in a luminometer upon the addition of 200 µl of 1% (v/v) H_2O_2 in 0.4 N HNO_3 followed immediately with alkalination of the solution with the immediate addition of (200 uℓ) 1M NaOH. The results are reported in relative light units (RLU), which is a measure of the number of photons emitted by the chemiluminescent label. Results are shown in Table 1 below.

TABLE 1

Comparison of Lyophilized Enzymes Stored at 25°C for 22 days with Unlyophilized Enzymes

	RNA Target		Negative Control	
	600 units MMLV-RT and 400 units of T7 polymerase	900 Units MMLV-RT and 400 units of T7 polymerase	600 units MMLV-RT and 400 units of T7 polymerase	900 Units MMLV-RT and 400 units of T7 polymerase
Liquid MMLV-RT and Liquid T7 RNA polymerase	321329	428872	1868	5630
Lyophilized MMLV-RT and Liquid T7 RNA polymerase	301253	463561	1681	1684
Liquid MMLV-RT and Lyophilized T7 RNA polymerase	549204	343582	1366	1545
Lyophilized MMLV-RT and Lyophilized T7 RNA polymerase (Separately Lyophilized)	415080	493779	1352	1374
Co-Lyophilized MMLV-RT and T7 RNA polymerase	677531 (900 units MMLV-RT)	654359	1376 (900 units MMLV-RT)	1296

[0052] These results indicate that the co-lyophilized MMLV-RT and T7 RNA polymerase caused amplification of the RNA Gene 10 target more effectively than in reaction mixtures with either enzyme preparation paired with a liquid enzyme preparation of the other enzyme, or where both enzymes were unlyophilized. The was no significant diminution in the ability of any of the lyophilized enzyme preparations to catalyze amplification as compared to the liquid enzymes. Thus, the results also demonstrate that each enzyme can be effectively stabilized by storage in a dried state in the presence of trehalose, either alone or together. Because nucleic acid amplification under these conditions depends on the presence of all three of the enzymatic activities of reverse transcriptase (RNA-directed DNA polymerase, DNA-directed DNA polymerase and RNAse H), the assay is an effective indication both that these activities are effectively stabilized by the present method and that the activities remain coordinated in such a way as to promote nucleic acid amplification.

[0053] Additional experiments showed that reverse transcriptase can be lyophilized in the presence of sucrose rather than trehalose under similar conditions; trehalose appeared to be slightly superior to sucrose as a cryoprotectant sta-

bilizing agent. (See Example 6.)

b. Lyophilization of Reverse Transcriptase and T7 RNA Polymerase in the presence of Non-Ionic Detergent

[0054] Reverse transcriptase and RNA polymerase were co-dialyzed and lyophilized in the presence of a non-ionic detergent in order to attempt to minimize precipitation of protein during the lyophilization procedure while maintaining the enzymatic activity dialysis of the enzymes. Six dialysis mixtures were prepared containing 0%, 0.01%, 0.05%, 0.1%, 0.2%, and 0.5% TRITON® X-102 in a dialysis buffer. The dialysis buffer contained 20 mM HEPES, 0.1 M NaCl, 0.1 mM EDTA, 5 mM NALC, 0.1 mM zinc acetate and 0.2 M trehalose. Final volume of each dialysis mixture was 250 ml. Four hundred sixty seven microliters of each buffer was combined with 46 μl MMLV-RT (2900 units/μl) and 74 μl T7 RNA polymerase (800 units/μl) for a starting volume for each dialysate of 587 μl. The samples were dialyzed against 60 ml of the corresponding buffer at 4°C with three changes of the same volume of buffer. Following the third buffer change, a precipitate was seen in the samples containing 0%, 0.01%, and 0.05% TRITON® X-102; no such precipitate was seen in the samples containing 0.1%, 0.2% or 0.5% TRITON® X-102.

[0055] After dialysis, the volume of each dialysate was measured and the calculated enzyme concentrations adjusted accordingly. Each sample was divided into 4 vials, with each vial containing 24,750 units of MMLV-RT and 11,000 units of T7 RNA polymerase. Lyophilization was performed as above. The appearance of the detergent-containing lyophilizates after drying was indistinguishable from lyophilizates prepared in the absence of TRITON® X-102. Following lyophilization, the vials were stored at 4°C and 55°C for 32 days.

[0056] The effect of the non-ionic detergent on the activity of the enzymes was assessed in an amplification assay using the T7 Gene 10 amplification system. Each lyophilized enzyme preparation was rehydrated in Reconstitution Buffer; 900 units of MMLV-RT and 400 units of T7 RNA polymerase were assayed in each reaction mixture. RNA Gene 10 transcripts (100 copies per reaction) were used as the target nucleic acid. The assay was conducted as described above unless expressly indicated otherwise. Results are reported in RLU.

TABLE 2

Stability of Lyophilized Enzymes Upon 32 Days' storage in the Presence of Detergent						
	Stored at 4°C			Stored at 55°C		
Sample*	RNA target (Duplicates)		No Target	RNA Target (Duplicates)		No Target
A	1612901	1317601	1543			
B	1151828	1146113	1700	791757	320417	1701
C	1286845	1219888	1544	1190527	905066	1690
D	1215264	1205790	1513	1251635	1388493	1513
E	1208586	1418260	1545	1245880	1052251	1591

* Sample A = Unlyophilized enzymes stored at -20°C.
Sample B = Lyophilized enzymes in 0% TRITON® X-102.
Sample C = Lyophilized enzymes in 0.1% TRITON® X-102.
Sample D = Lyophilized enzymes in 0.2% TRITON® X-102.
Sample E = Lyophilized enzymes in 0.5% TRITON® X-102.

[0057] These results demonstrate that a non-ionic detergent such as TRITON® X-102 can effectively prevent the formation of a protein precipitate after dialysis of MMLV-RT or T7 RNA polymerase. The results also show that TRITON® X-102 dos not have a deleterious effect upon amplification of the target nucleic acid, and may even act to better stabilize the enzyme activities when the lyophilized enzymes are stored at elevated temperatures over time. The detergent does not cause an increase in the background luminescence in this assay. These results also demonstrate that even the sample lyophilized in the absence of detergent (Sample B) remains approximately as active as non-lyophilized enzymes. The results indicate further that when the lyophilized enzyme preparation is stored at elevated temperature for a prolonged period of time the lyophilized enzyme preparation does not experience detectable diminution in activity.

[0058] It will be clear to one of skill in the art that these results immediately suggest that other non-ionic detergents such as, without limitation, detergents of the BRIJ series, the TWEEN series, other detergents of the TRITON series, and the TERGITOL series may be easily screened as indicated above for their ability to maintain the dried proteins in a soluble state during lyophilization without having an adverse effect on enzyme activity.

Example 2: Co-Lyophilization of Reverse Transcriptase and RNA Polymerase with Amplification Reagents

[0059] Moloney Murine Leukemia Virus reverse transcriptase and T7 RNA polymerase enzyme preparations were kept at -20°C in a storage buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) NP®-40 or 0.1% (v/v) TRITON® X-100 and 50% (v/v) glycerol prior to drying.

[0060] In preparation for lyophilization, 3 x 10^6 units of MMLV RT and 1.3 x 10^6 units of T7 polymerase (2.5 ml of each preparation) were combined and dialyzed against at least 50 volumes of a buffer containing 20 mM HEPES(pH 7.5), 5 mM NALC, 0.1 mM EDTA, 0.1 mM zinc acetate, 0.2% (v/v) TRITON® X-102, and 0.2 M trehalose using dialysis membranes with a molecular weight cutoff of 12,000 Daltons at 2-8°C with three changes of the same volume of buffer for at least 8 hours between each buffer change.

[0061] Twenty milliliters of the dialyzed enzyme preparation was combined with 60 ml of an Amplification Reagent containing 10.0 mM spermidine, 250 mM imidazole/150 mM glutamic acid (pH 6.8), 99 mM NALC, 12.5% (w/v) PAP, 12.5 mM each of rCTP and rUTP, 31.2 mM each of rATP and rGTP, and 10.0 mM each of dCTP, dGTP, dATP and dTTP (6:2 volume ratio). Additional experiments have shown that the reagents may be combined in a 7:1 volume ratio (Amplification Reagent to enzyme preparation) without significantly different results. Theoretically, the dialyzed enzyme preparation and the Amplification Reagent may be combined in equal proportions; determination of an appropriate ratio of Amplification Reagent to enzyme is well within the ability of the skilled artisan.

[0062] The final composition of the combined enzyme:Amplification Reagent formulation prior to lyophilization was: 2.7 x 10^5 units of MMLVRT and 1.2 x 10^6 of T7 polymerase 6 X 10^6 units of each enzyme, 5.0 mM HEPES (pH 6.8 to 7.0), 0.025 mM EDTA, 0.025 mM zinc acetate, 10.0 mM spermidine, 187.5 mM imidazole, 112.5 mM glutamic acid, 75.6 mM NALC, 0.05% (v/v) TRITON' X-102, 9.4% (w/v) PAP (average MW 40,000 Daltons), 0.05 M trehalose, 9.4 mM each of rCTP and rUTP, 23.4 mM each of rATP and rGTP, and 7.5 mM each of dCTP, dGTP, dATP and dTTP.

[0063] Eight hundred microliters of the combined enzyme:Amplification Reagent preparation (hereafter Enzyme: Amplification Reagent) were placed into each individual glass vial for lyophilization (approximately 39,000 units of total enzymes per vial). Lyophilization was conducted as follows in Example 1. After lyophilization, the vials were then treated as indicated in the following examples.

Example 3: Amplification Activity Assay of Lyophilized Reaaent

[0064] Freshly lyophilized preparations of reverse transcriptase, RNA polymerase, and Amplification Reagent were incubated at 25°C, 35°C and 45°C for various times, ranging from 3 to 61 days. All vials were prepared identically from the same preparation. At the indicated time points vials containing the lyophilized reagents were removed from elevated temperature and stored at -30°C until the last samples had been collected. Samples representing the "zero" time for each temperature were stored at -30°C for the entire experimental time period.

[0065] When the vials from the last time point had been collected all samples were rehydrated in 1.5 ml of Reconstituting Reagent (0.01% (v/v) TRITON® X-102, 41.6 mM $MgCl_2$, 1 mM $ZnC_2H_3O_2$, 10% (v/v) glycerol, 0.3% (v/v) ethanol, 0.02% (w/v) methyl paraben, and 0.01% (w/v) propyl paraben) and the contents of each vial assayed for the ability to cause nucleic acid amplification.

[0066] Activity in a model amplification system was measured in the following way in this example. Each amplification reaction mixture contained 500 copies of a double-stranded DNA restriction fragment from a plasmid containing part of the hepatitis B virus genome as the target nucleic acid (a PUC plasmid containing a 2.6kb fragment of the hepatitis B virus genome). The target DNA was diluted in 20 µl of either water or human serum. Negative controls were made in the same way, but without target DNA. This was added to 20 µl of a 2X primer solution; the final composition of this solution was 0.1 N KOH, 17.5 mM EGTA, 25 mM imidazole, 25 mM glutamic acid, 0.025% (w/v) phenol red, and 0.3 µM of each of two primers in a total volume of 40 µl. The first primer ((-) sense) consisted of a 3' target-binding nucleotide sequence region complementary to the (+) sense strand of the DNA target and a 5' non-complementary region was situated downstream from a 5' non-complementary region having the nucleotide sequence of the promoter for T7 RNA polymerase. The second primer ((+) sense) had a nucleotide sequence consisting of a target-binding region complementary to the other ((-) sense) DNA strand.

[0067] Each 40 µl reaction mixture was incubated at about 95°C to denature the double-stranded DNA target. The reaction was then cooled to room temperature for 5 minutes and neutralized with 10 µl of a buffer containing 330 mM imidazole and 200 mM glutamic acid. Had the target nucleic acid been RNA rather than DNA this denaturation step would not be necessary.

[0068] Fifty microliters of each reconstituted Enzyme: Amplification Reagent was given to 50 µl of the denatured, neutralized DNA reaction mixture, which was then incubated at 37°C for 3 hours. Each reaction was terminated by the addition of 20 µl (40 units) of RNAse-fr e DNAse I.

[0069] The relative amplification of each reconstituted Enzyme:Amplification Reagent was determined by using the homogeneous protection assay (HPA) described in Arnold & Nelson, U.S. Patent No. 5,283,174; it will be understood

by those of skill in the art that other assay methods employing different detection means, such as radioactive labels, may be used. Each amplification reaction was given 100 µl of a solution of 10 mM lithium succinate (pH 5.0), 2% (w/v) lithium lauryl sulfate, 1 mM mercaptoethanesulfonic acid, 0.3% (w/v) PAP-40, 230 mM LiOH, 1.2 M LiCl, 20 mM EGTA, 20 mM EDTA, 100 mM succinic acid (pH 4.7) and 15 mM 2,2'-dipyridyl disulfide containing approximately 75 femtomoles (fmol) of an acridinium ester-labeled oligonucleotide probe ((+) sense) designed to be complementary to the amplified RNA amplicons. Each tube was mixed, incubated at 60°C for 20 minutes, and then allowed to cool. Each reaction mixture was given 300 µl of a solution containing 0.6 M sodium borate (pH 8.5), 1% (v/v) TRITON® X-100 and 182 mM NaOH and incubated for 6 minutes at 60°C to destroy label unassociated with hybridized probe.

[0070] The reaction mixtures were cooled for 5 minutes, and the remaining chemiluminescence was measured in a luminometer (LEADER® Gen-Probe Incorporated, San Diego, CA) after an automatic injection of 200 µl 0.1% (v/v) H_2O_2, 0.1 mM nitric acid, followed immediately by an injection of 1.0 N NaOH. The amount of subsequently emitted light is reported in Relative Light Units (RLU). Under these conditions the background level of light emission was in the range of about 2000 to 4000 RLU.

[0071] The results were recorded and tabulated for each temperature of storage (25°C, 35°C and 45°C) as indicated below. Each sample was assayed in triplicate and averaged. This average was used to plot the data for each temperature graphically. Figure 1 corresponds to Table 3, Figure 2 to Table 4, and Figure 3 to Table 5.

TABLE 3

Stability of Lyophilized Enzyme:Amplification Reagent Storage Temperature 25°C

Days of Storage	0	11	16	20	30	40	61
Reagents without DNA Target (RLU)	2053 2130 2148	1911 1590 1752	1524 1561 2037	2188 1990 1606	1851 1847 1923	1548 1726 2382	1972 1655 1538
Average RLU	2110	1751	1707	1928	1874	1885	1722
Reagents with DNA Target (RLU)	1562029 1756224 1070164	2105440 1903081 1492458	1248988 1509929 1944566	2129935 2363198 1922529	1927067 1422699 1274124	1417883 1601071 1889588	1486111 1290950 1210344
Average RLU	1462806	1833659	1567828	2138554	1541297	1636181	1329135
Reagents in Human Serum, No DNA Target (RLU)	8437 3902 3534	2904 2893 3003	2660 2993 2768	3044 3152 2951	2919 2971 2379	2465 3089 2958	2946 3473 3686
Average RLU	5291	2933	2807	3049	2756	2837	3368
Reagents in Human Serum, with DNA Target (RLU)	1955525 2255411 2282281	2282336 2204415 2206778	2282171 1860043 1903519	1760428 1992765 2093235	2034705 2101999 2064041	1936366 1770109 1811820	1643624 1762360 1622750
Average RLU	2164406	2231176	2015244	1948809	2066915	1839432	1676245

TABLE 4

Stability of Lyophilized Enzyme/Amplification Reagent Storage Temperature 35°C							
Days of Storage	0	3	9	16	21	50	61
Reagents without DNA Target (RLU)	2429 2203 1996	17989 1775 1891	1768 1649 1840	1878 1919 2043	2378 2330 1995	1430 1411 1338	1559 1566 1692
Average RLU	2209	7218	1752	1947	2234	1393	1606
Reagents with DNA Target (RLU)	1173260 1580018 1389614	2310573 2136598 2303010	2186899 2119044 1568334	1559681 1385165 1632416	1876363 1919833 1979406	1458120 1932847 1343433	1366068 1443874 1421081
Average RLU	1380964	2251060	1958092	1525754	1925201	1578133	1410341
Reagents in Human Serum, No DNA Target (RLU)	4819 4779 24541	3298 9577 3349	3608 3200 3114	3575 3535 3712	2912 3422 3151	3074 3044 3027	3836 4160 3901
Average RLU	11380	5408	3307	3607	3162	3048	3966
Reagents in Human Serum, with DNA Target (RLU)	1946881 2158003 2110796	2228745 2289829 2286956	2233566 2303812 2179206	2087936 2163922 2152655	1984355 2192597 2121658	2255784 2147927 2087549	1873070 1789954 2049762
Average RLU	2071893	2268510	2238861	2134838	2099537	2163753	1904262

TABLE 5

Stability of Lyophilized Enzyme/Amplification Reagent Storage Temperature 45°C					
Days of Storage	0	6	11	16	33
Reagents without DNA Target (RLU)	2508 2250 2159	1613 1872 1903	1687 1781 2206	2626 2027 2056	1594 1596 1661
Average RLU	2306	1796	1891	2236	1617
Reagents with DNA Target (RLU)	1431296 1329706 1288191	1097084 949892 798877	975001 758705 1242188	1320113 939417 972442	1017853 1368153 1015174
Average RLU	1349731	948618	991965	1077324	1133727
Reagents in Human Serum, No DNA Target (RLU)	3554 3109 4239	3375 4452 2960	3011 3119 3382	3068 3559 3381	3183 3115 2826
Average RLU	3634	3596	3171	3336	3041
Reagents in Human Serum, with DNA Target (RLU)	1663770 1677985 1747637	1850263 1868747 2016609	1691590 1684565 1646303	1691372 1709387 1765393	1615426 1913706 1799445
Average RLU	1696464	1911873	1674153	1722051	1776192

[0072] These data show that the co-lyophilized Enzyme:Amplification Reagent prepared in accordance with the meth-

od herein described retains all four of the enzymatic activities (RNA-directed DNA polymerase, DNA-directed DNA polymerase, RNAse H, and RNA polymerase) necessary to achieve nucleic acid amplification according to the transcription-mediated amplification method employed. Additionally, the data indicate that there is no noticeable deleterious effect on the nucleotide triphosphates or any other component of the Amplification Reagent when the reagent is co-lyophilized with reverse transcriptase and RNA polymerase.

[0073] These results also show that the enzymatic activities of reverse transcriptase and RNA polymerase enzymatic activities are not significantly inhibited when the amplification reaction is performed in the presence of a complex biological sample, such as human serum. Hence, the lyophilized amplification reagent appears to be suitable for use in conjunction with samples such as those obtained in clinical diagnostic settings.

[0074] The data can be interpreted in a number of ways; one of the more useful means of interpretation utilizes a form of the Arrhenius equation to predict the stability of the composition over an even greater time than actually tested. The Arrhenius equation is commonly used by those of skill in the art to predict the rates of chemical reactions and the stability of various thermolabile compounds as a function of temperature.

[0075] As utilized herein, the Arrhenius equation assumes a first order reaction of enzyme (or reagent) inactivation wherein an active enzyme or reagent has a single rate of inactivation at a given temperature and a single mechanism of inactivation at all tested temperatures. The equation utilized by the Applicant is:

$$\ln(k_2/k_1) = (-E_a/R)\ ((T_2-T_1) / (T_2 \times T_1))$$

where k_2 equals the rate constant at the experimental temperature (°K), k_1 equals the rate constant for the reaction at a reference temperature, E_a equals the activation energy of the reaction, R equals the gas constant (1.987 cal/°K-mole), T_1 equals the reference temperature (e.g., 298.16°K (25°C)), and T_2 equals the experimental temperature (expressed in °K).

[0076] If Eg is assumed to be 15,000 cal/mole and the reference and experimental temperatures are known, then a ratio of the rate constants k_2/k_1 can be determined. In the simple case where both the reference and experimental temperatures are 25°C, the ratio of these constants is 1 since the constants are identical. If the experimental temperature is 35°C and the reference temperature is 25°C, the predicted ratio will be 2.27. If the experimental temperature is 45°C and the reference temperature is 25°C, the predicted ratio will be 4.91. Using the same equation, if the reference temperature is 5°C and the experimental temperature is 45°C, the ratio is 30.33.

[0077] The rate constant ratios can be considered the "decomposition ratio" of the experimental storage time to the normal storage time, whether this time is expressed in hours, days, weeks, etc. Therefore, if the lyophilized enzyme/amplification reagent decomposes to 90% of its original potency in 30 days at 45°C, the Arrhenius equation predicts that it would take 147.3 (30 x 4.91) days at 25°C for the activity to be similarly reduced.

[0078] Thus, the data demonstrate that the combined components of the lyophilized preparation do not noticeably lose their ability to support amplification in "real time", even after 30 days at 45°C. Moreover, by utilizing the Arrhenius equation the same data predict that the reagents would not suffer a significant loss in activity if the lyophilized reagent was actually stored for almost 5 months at 25°C or for 2.5 years (30.33 x 30 days) at 5°C prior to use.

[0079] The Applicant presents these methods of data analysis as an aid to the understanding of the present invention, and does not wish to be limited or bound by theoretical considerations. The actual stability of the compositions of the present invention may vary from the predictions of the Arrhenius equation, which provides general guidance toward predicting the stability of the lyophilized reagents.

Example 4: <u>T7 RNA Polymerase Assay of Lyophilized Reagent</u>

[0080] The lyophilized Enzyme:Amplification Reagent prepared in Example 2 was incubated at 35°C for 0, 3, 9, 16, 21 and 30 days. At each of these time points vials were removed from the stress temperature and stored at -30°C until the last samples had been collected.

[0081] RNA polymerase activity was measured by reconstituting each aliquot of lyophilized reagent in 1.5 ml of Reconstituting Buffer (0.01% (v/v) TRITON® X-100, 41.6 mM $MgCl_2$, 1 mM $ZnC_2H_3O_2$, 10% (v/v) glycerol, 0.3% (v/v) ethanol, 0.02% (w/v) methyl paraben, and 0.01% (w/v) propyl paraben). The reagent was then diluted 100-fold, 200-fold and 400-fold in a solution containing 20 mM HEPES (pH 7.5), 5 mM NALC, 0.1 mM EDTA, 0.1 mM $ZnC_2H_3O_2$, 0.1 M NaCl and 0.2% (v/v) TRITON® X-102. A reaction pre-mix was made up separately, containing 22 mM $MgCl_2$, 7.8 mM each of ATP and GTP, 2.5 mM each of CTP and UTP, 62.5 mM Tris (pH 7.5), 2.5 mM spermidine and 0.5 nanomoles of a target nucleic acid. The target was a linearized pUC T7G10 plasmid having a T7 promoter positioned immediately upstream from bacteriophage T7 Gene 10. This plasmid was derived from plasmid pGEMEX-1 (Promega Corporation, Madison, WI).

[0082] The reaction pre-mix was divided into 40 μl aliquots, and each aliquot was incubated for 3 minutes at 37°C.

Ten microliters of each dilution of the Enzyme:Amplification Reagent was added to the warmed pre-mix tubes and incubated for 20 minutes at 37°C. Fifty microliters of a solution of 10mM lithium succinate, 2% (w/v) lithium lauryl sulfate, 1 mM mercaptoethanesulfonic acid, 0.3% (w/v) PAP-40, 230 mM LiOH, 1.2 M Lid, 20 mM EGTA, 20 mM EDTA, 100 mM succinic acid (pH 4.7) and 15 mM 2.2'-dipyridyl disulfide containing approximately 75 femtomoles of an acridinium ester labeled Gene 10 oligonucleotide probe ((-) sense) designed to be complementary to the transcriptional products was added to each tube. A standard sample containing 10 femtomoles (fmol) of single-stranded DNA complementary to the Gene 10 probe was included in the HPA step to quantitate the amount of RNA produced in the experimental reaction mixtures. Hybridization was performed essentially as in Example 2, except that the hybridization volumes were half as large. Following degradation of the unhybridized label, the remaining acridinium ester was reacted and the emitted light measured in a luminometer as RLU.

[0083] The raw data was converted to units of RNA polymerase activity per µl as follows. The raw RLU obtained for the positive control reaction was subtracted from the RLU obtained in the negative control (no target DNA). This figure represents the net amount of emitted light obtained when 10 fmol of RNA are in the sample, and can be expressed as RLU/fmol RNA. Likewise, the RLU obtained for each sample can be subtracted from the background luminescence (RLU per 20 minutes). When this figure is divided by the figure obtained for the standard (RLU per fmol RNA) the result is the number of fmol RNA produced in each reaction per 20 minutes. Because 1 unit RNA polymerase activity was defined as the production of 1 fmol RNA in 20 minutes under the assay conditions, this figure is also the number of units of RNA polymerase activity in each 10 µl volume of enzyme originally added.

[0084] The data obtained from these reactions were first plotted for each time of storage at 35°C by expressing fmol of RNA produced as a function of the number of microliters of the original 1.5 ml reconstituted Enzyme:Amplification Reagent represented in each experimental tube. A simple linear function was described. When the data had been plotted, a best-fit line for the data obtained for each time point was calculated; the slope of this curve was expressed as units of T7 polymerase activity per microliter. When the "zero time" time point is considered as 100% activity, the calculated units of T7 RNA polymerase for each remaining time point was expressed as percent activity remaining.

[0085] Figure 4 is a plot of the number of units of T7 RNA polymerase per microliter in the lyophilized Enzyme: Amplification Reagent as a function of the number of days of storage at 35°C. The results indicate that little if any decrease in T7 RNA polymerase occurs over the 30 day 35°C incubation period.

Example 5: Reverse Transcriptase Assay of the Lyophilized Reagent

[0086] The activity of lyophilized MMLV reverse transcriptase incubated for 3, 9, 16, 21 and 30 days at 35°C was assayed as follows. Individual vials were removed from the stress temperature at the indicated times and stored at -30°C until the last samples had been collected.

[0087] Each vial was reconstituted in 1.5 ml reconstitution buffer and diluted 100 fold, 200-fold, and 400-fold as in Example 4. A separate reverse transcriptase pre-mix mixture was made containing 5 mM $MgCl_2$, 30 mM KCl, 0.25 mM each of dATP, dTTP, dCTP, and dGTP, 62.5 mM Tris (pH 7.5), 2.5 mM spermidine, 3.75 nM target RNA, and 750 nM of an amplification primer. The target RNA was the T7 Gene 10 RNA transcripts generated in Example 4. The primer was an oligonucleotide 22 bases in length designed to hybridize to a region near the 3' end of the target RNA. Ten microliters of the enzyme dilutions were each added to 40 µl of the reaction pre-mix on ice. The reactions were conducted by incubation at 37°C for 15 minutes. Each reaction was terminated with the addition of 50 µl of an acridinium ester-labeled hybridization probe. The probe was designed to be complementary to the newly synthesized Gene 10 cDNA.

[0088] Detection by HPA was conducted as described in Example 3. Results were measured in RLU.

[0089] This assay measured the RNA-directed DNA polymerase activity and the RNAse H activity of the MMLV reverse transcriptase. The latter activity is indirectly measured, since without degradation of the RNA strand of the RNA:DNA hybrid produced by extension of the Gene 10 primer, the probe would not be able to hybridize to the cDNA.

[0090] One unit of these combined enzymatic activities was defined as the detection of 1 fmol cDNA in 15 minutes under the reaction conditions described above. Calculation of the units of enzyme activity remaining at each time point and dilution was performed as in Example 4 using 10 fmol of the amplified cDNA as a standard.

[0091] Figure 5 is a plot of the number of units of RT activity per microliter in the lyophilized Enzyme:Amplification Reagent as a function of the number of days of storage at 35°C. The results indicate that little if any decrease in RT activity occurs over the 30 day 35°C incubation period.

Example 6: Co-Lyophilization of Reverse Transcriptase and RNA Polymerase with Nucleotides and Primers.

[0092] The preceding examples have illustrated the preparation and use of a single reagent containing a desiccated preparation of RNA polymerase and reverse transcriptase together with nucleotide triphosphates and co-factors necessary for nucleic acid amplification. It will be clear to one of skill in the art that, given the ability of such a "single vial"

reagent to amplify nucleic acids after prolonged storage at raised temperatures, it should easily be possible to include the amplification primer(s) in the lyophilized preparation so as to reduce the number of steps in methods of using such a reagent, and to reduce the number of containers in a kit for nucleic acid amplification from three (for example, lyophilized Enzyme:Amplification Reagent, primers and Reconstitution Reagent) to two (for example, lyophilized Enzyme/ primer/Amplification Reagent and Reconstitution Reagent).

[0093] Such a preparation is useful when the amplification reaction does not make use of temperatures which will denature one or both of the enzymes, such as when the initial target nucleic acid is RNA and the amplification method is an isothermal one, for example as in Kacian & Fultz, PCT Publication No. WO91/01384 or Kacian et al., PCT Publication No. WO93/22461.

Example 7: Lyophilization of Reverse Transcriptase with Sucrose

[0094] Applicant has also discovered that sucrose, (for example, at a concentration of 0.2 M), can be used as a cryoprotectant stabilizing agent in the lyophilization of reverse transcriptase; the stabilizing effect of sucrose appears to be good; compared to a standard liquid solution containing MMLV-RT and stored for the same period of time in 50% (v\v) glycerol at -20°C the preparation lyophilized in 0.2 M sucrose maintained 93% of the activity of the standard MMLV-RT preparation following storage of the lyophilizate for 30 days at 4°C. A similarly treated lyophilizate containing 0.2 M trehalose rather than sucrose showed an average of 105% of the activity of the standard under the same conditions.

SEQUENCE LISTING

[0095]

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: GEN-PROBE INCORPORATED
(B) STREET: 9880 Campus Point Drive
(C) CITY: San Diego
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(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 92121

(ii) TITLE OF INVENTION: STABILIZED ENZYME COMPOSITIONS FOR NUCLEIC ACID AMPLIFICATION

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA: APPLICATION NUMBER: EP

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTTAATAC GACTCACTAT AGGGAGAGAG AAGTGGTCAC GGAGGTAC 48

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: CDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATGACTGGT GGACAGCAAA TG 22

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGCTGGAGA TAAACTGGCG TTGTTC 26

Claims

1. A method for preparing a stabilized enzyme composition in a single vial comprising the steps of:

a) providing a solution comprising

i) an active enzyme composition comprising a mixture of a reverse transcriptase and an RNA polymerase, and
ii) a stabilizing agent, said stabilizing agent consisting of a non-reducing disaccharide or polyvinylpyrro-

lidone,

b) freezing the solution of (a),
c) sublimating a solvent fraction of said frozen solution by application of a vacuum, thereby forming a lyophilizate comprising said active enzyme composition and said stabilizing agent.

2. The method of claim 1 wherein said stabilizing agent consists of trehalose.

3. The method of claim 1 wherein said non-reducing disaccharide is trehalose.

4. The method of claim 1 wherein said stabilizing agent consists of polyvinylpyrrolidone.

5. The method of claim 1 wherein said solution also comprises deoxyribonucleotide triphosphates, ribonucleotide triphosphates, metal salts and cofactors sufficient to permit both DNA polymerization and RNA transcription in a single solution when said lyophilizate is reconstituted and combined with appropriate nucleic acid substrates and reactants.

6. The method of claim 5 wherein said solution also comprises at least one oligonucleotide amplification primer.

7. The method of claim 5 wherein said enzyme composition retains at least 70% of its ability to amplify a target nucleic acid when said lyophilizate is stored at room temperature for two months.

8. The method of claim 5 wherein said enzyme composition retains at least 70% of its ability to amplify a target nucleic acid when said lyophilizate is stored at 35°C for two months.

9. The method of claim 5 wherein said enzyme composition retains at least 70% of its ability to amplify a target nucleic acid when said lyophilizate is stored at 45°C for two months.

10. The method of claim 5 wherein said enzyme composition retains at least 70% of its ability to amplify a target nucleic acid when said lyophilizate is stored for 55°C for two months.

11. A composition produced by the method of any one of the preceding claims.

12. A composition for amplification of a target nucleic acid comprising a single lyophilizate having

a) an effective amount of an RNA-directed DNA polymerase activity, a DNA-directed DNA-polymerase activity, an RNAse H activity, and a DNA-directed RNA polymerase activity wherein the RNA-directed DNA polymerase activity, DNA-directed DNA polymerase activity, and RNAse H activity are provided by one or more separate enzymes,
b) a stabilizing agent that consists of either a non-reducing disaccharide or polyvinylpyrrolidone,
c) deoxyribonucleotide triphospates and ribonucleotide triphosphates,
d) metal salts, and
e) a reducing agent, wherein when said lyophilizate is reconstituted by addition of an aqueous solvent the resulting solution will amplify a single-stranded RNA molecule having a target nucleotide sequence region upon addition of said RNA molecule and one or more suitable oligonucleotide primers to said solution and incubating the solution at a temperature sufficient to promote said enzymatic activities.

13. The composition of claim 12 wherein said one or more suitable primers is comprised in the lyophilizate.

14. The composition of claim 13, further comprising

f) a buffer, but provided said composition does not contain a carboxylic acid.

15. The composition of claim 12 or 13 wherein said RNA-directed DNA polymerase activity, DNA-directed DNA-polymerase activity, and RNAse H activity are provided by a recombinant retroviral reverse transcriptase, and said DNA-directed RNA polymerase activity is provided by a bacteriophage RNA polymerase.

16. The composition of claim 15 wherein said reverse transcriptase is derived from Moloney Murine Leukemia Virus.

17. The composition of claim 15 wherein said RNA polymerase is derived from T7 bacteriophage.

18. The composition of claim 15 wherein said stabilizing agent is polyvinylpyrrolidone.

19. The composition of claim 14 wherein said stabilizing agent is trehalose.

20. A kit for amplification of a target nucleic acid comprising a reverse transcriptase and an RNA polymerase combined in a single lyophilized formulation together with a cryoprotectant stabilizing agent that consists of either non-reducing disaccharide or polyvinylpyrrolidone, wherein upon rehydration of said lyophilized formulation and addition of the target nucleic acid in the presence of oligonucleotide primers, some or all of said target nucleic acid will be amplified.

21. The kit of claim 20 wherein said lyophilized formulation further comprises metal salts and nucleotide triphosphates.

22. The kit of claim 21 further comprising at least one oligonucleotide amplification primer.

23. The kit of any of claims 20 to 22 wherein said stabilizing agent is trehalose.

24. The kit of any of claims 20 to 22 wherein said stabilizing agent is polyvinylpyrrolidone.

Patentansprüche

1. Ein Verfahren zum Herstellen einer stabilisierten Enzymzusammensetzung in einem einzelnen Fläschchen, das die Schritt umfasst:

    a) Bereitstellen einer Lösung, die umfasst

        i) eine aktive Enzymzusammensetzung, die ein Gemisch einer reversen Transkriptase und einer RNA Polymerase umfasst, und
        ii) ein stabilisierendes Agens, wobei das stabilisierende Agens aus einem nicht reduzierenden Disaccharid oder Polyvinylpyrrolidon besteht,

    b) Einfrieren der Lösung von (a),
    c) Sublimieren einer Lösungsmittelfraktion der gefrorenen Lösung durch Anlegen eines Vakuums, wodurch ein Lyopyhilisat gebildet wird, das die aktive Enzymzusammensetzung und das stabilisierende Agens umfasst.

2. Das Verfahren nach Anspruch 1, worin das stabilisierende Agens aus Trehalose besteht.

3. Das Verfahren nach Anspruch 1, worin das nicht reduzierende Disaccharid Trehalose ist.

4. Das Verfahren nach Anspruch 1, worin das stabilisierende Agens aus Polyvinylpyrrolidon besteht.

5. Das Verfahren nach Anspruch 1, worin die Lösung zudem Deoxyribonukleotid-Triphosphate, Ribonukleotid-Triphosphate, Metallsalze und Kofaktoren umfasst, die ausreichend sind, um sowohl die DNA-Polymerisation als auch die RNA-Transkription in einer einzelnen Lösung zu gestatten, wenn das Lyophilisat rekonstituiert und mit geeigneten Nukleinsäuresubstraten und Reaktionspartnern kombiniert wird.

6. Das Verfahren nach Anspruch 5, worin die Lösung zudem mindestens einen Oligonukleotid-Amplifikationsprimer umfasst.

7. Das Verfahren nach Anspruch 5, worin die Enzymzusammensetzung mindestens 70% ihrer Fähigkeit behält, eine Zielnukleinsäure zu amplifizieren, wenn das Lyophilisat bei Raumtemperatur für zwei Monate gelagert wird.

8. Das Verfahren nach Anspruch 5, worin die Enzymzusammensetzung mindestens 70% ihrer Fähigkeit behält, eine Zielnukleinsäure zu amplifizieren, wenn das Lyophilisat bei 35°C für zwei Monat gelagert wird.

9. Das Verfahren nach Anspruch 5, worin die Enzymzusammensetzung mindestens 70% ihrer Fähigkeit behält, eine

Zielnukleinsäure zu amplifizieren, wenn das Lyophilisat bei 46°C für zwei Monate gelagert wird.

10. Das Verfahren nach Anspruch 5, worin die Enzymzusammensetzung mindestens 70% ihrer Fähigkeit behält, eine Zielnukleinsäure zu amplifizieren, wenn das Lyophilisat bei 55°C für zwei Monate gelagert wird.

11. Eine Zusammensetzung, erzeugt durch das Verfahren nach einem der vorhergehenden Ansprüche.

12. Eine Zusammensetzung für die Amplifikation einer Zielnukleinsäure, die ein einzelnes Lyophilisat umfasst, welches besitzt

a) eine wirksame Menge einer RNA-gerichteten DNA-Polymeraseaktivität, einer DNA-gerichteten DNA-Polymeraseaktivität, einer RNAse H Aktivität und einer DNA-gerichteten RNA-Polymeraseaktivität, worin die RNA-gerichtete DNA-Polymeraseaktivität, die DNA-gerichtete DNA-Polymeraseaktivität und die RNAse H Aktivität durch ein oder mehrere separate Enzyme bereitgestellt wird,
b) ein stabilisierendes Agens, das entweder aus einem nicht reduzierenden Disaccharid oder Polyvinylpyrrolidon besteht,
c) Deoxyribonukleotid-Triphosphate und Ribonukleotid-Triphosphate,
d) Metallsalze, und
e) ein reduzierendes Agens, worin, wenn das Lyophilisat durch Zugabe eines wässrigen Lösungsmittels rekonstituiert wird, die sich ergebende Lösung ein einzelsträngiges RNA-Molekül mit einem Zielnukleotid-Sequenzbereich nach Zugabe des RNA-Moleküls und eines oder mehrerer geeigneter Oliognukleotidprimer zu der Lösung und dem Inkubieren der Lösung bei einer Temperatur, die ausreichend ist, um die enzymatischen Aktivitäten zu fördern, amplifizieren wird.

13. Die Zusammensetzung nach Anspruch 12, worin ein oder mehrere geeignete Primer von dem Lyophilisat umfasst werden.

14. Die Zusammensetzung nach Anspruch 13, die weiter umfasst

f) einen Puffer, vorausgesetzt jedoch, dass die Zusammensetzung keine Carbonsäure enthält.

15. Die Zusammensetzung nach Anspruch 12 oder 13, worin die RNA-gerichtete DNA-Polymeraseaktivität, die DNA-gerichtete DNA-Polymeraseaktivität und die RNAse H Aktivität durch eine rekombinante retrovirale reverse Transkriptase bereitgestellt werden und die DNA-gerichtete RNA-Polymeraseaktivität durch eine RNA-Polymerase eines Bakteriophagen bereitgestellt wird.

16. Die Zusammensetzung nach Anspruch 15, worin die reverse Transkriptase aus dem Moloney Maus Leukämie Virus gewonnen wird.

17. Die Zusammensetzung nach Anspruch 15, worin die RNA-Polymerase aus dem T7 Bakteriophagen gewonnen wird.

18. Die Zusammensetzung nach Anspruch 15, worin das stabilisierende Agens Polyvinylpyrrolidon ist.

19. Die Zusammensetzung nach Anspruch 14, worin das stabilisierende Agens Trehalose ist.

20. Ein Kit für die Amplifikation einer Zielnukleinsäure, der eine reverse Transkriptase und eine RNA-Polymerase umfasst, die in einer einzelnen lyophilisierten Formulierung zusammen mit einem Kälteschutzmittel stabilisierenden Agens, das entweder aus einem nicht reduzierenden Disaccharid oder Polyvinylpyrrolidon besteht, kombiniert sind, worin nach der Rehydratation der lyophilisierten Formulierung und der Zugabe der Zielnukleinsäure in der Gegenwart von Oligonukleotidprimern, einige oder alle der Zielnukleinsäuren amplifiziert werden.

21. Der Kit nach Anspruch 20, worin die lyophilisierte Formulierung weiter Metallsalze und Nukleotid-Triphosphate umfasst.

22. Der Kit nach Anspruch 21, der weiter mindestens inen Oligonukleotid-Amplifikationsprimer umfasst.

23. Der Kit nach einem der Ansprüche 20 bis 22, worin das stabilisierende Agens Trehalose ist.

24. Der Kit nach einem der Ansprüche 20 bis 22, worin das stabilisierende Agens Polyvinylpyrrolidon ist.

Revendicati ns

1. Un procédé pour la préparation d'une composition enzymatique stabilisée dans un flacon unique comprenant les étapes consistant à :

   a) fournir une solution composée

      i) d'une composition enzymatique active comprenant un mélange d'une réverse transcriptase et d'une ARN polymérase, et
      ii) d'un agent stabilisateur, ledit agent stabilisateur étant composé d'un disaccharide non réducteur ou de polyvinylpyrrolidone,

   b) congeler la solution de a),
   c) sublimer une fraction du solvant de ladite solution congelée par application d'un vide, formant ainsi un lyophilisat comprenant ladite composition enzymatique active et ledit agent stabilisateur.

2. Le procédé selon la revendication 1 dans lequel ledit agent stabilisateur est composé de tréhalose.

3. Le procédé selon la revendication 1 dans lequel ledit disaccharide non réducteur est du tréhalose.

4. Le procédé selon la revendication 1 dans lequel ledit agent stabilisateur est composé de polyvinylpyrrolidone.

5. Le procédé selon la revendication 1 dans lequel ladite solution comprend également des désoxyribonucléotide-triphosphates, des ribonucléotide-triphosphates, des sels métalliques et des cofacteurs suffisants pour permettre la polymérisation de l'ADN et la transcription de l'ARN dans une solution unique lorsque ledit lyophilisat est reconstitué et combiné à des substrats d'acides nucléiques et des réactants appropriés.

6. Le procédé selon la revendication 5 dans lequel ladite solution comprend également au moins une amorce oligonucléotidique pour l'amplification.

7. Le procédé selon la revendication 5 dans lequel ladite composition enzymatique conserve au moins 70% de sa capacité à amplifier un acide nucléique cible lorsque ledit lyophilisat est conservé à température ambiante pendant deux mois.

8. Le procédé selon la revendication 5 dans lequel ladite composition enzymatique conserve au moins 70% de sa capacité à amplifier un acide nucléique cible lorsque ledit lyophilisat est conservé à 35°C pendant deux mois.

9. Le procédé selon la revendication 5 dans lequel ladite composition enzymatique conserve au moins 70% de sa capacité à amplifier un acide nucléique cible lorsque ledit lyophilisat est conservé à 45°C pendant deux mois.

10. Le procédé selon la revendication 5 dans lequel ladite composition enzymatique conserve au moins 70% de sa capacité à amplifier un acide nucléique cible lorsque ledit lyophilisat est conservé à 55°C pendant deux mois.

11. Une composition produite par le procédé selon l'une quelconque des revendications précédentes.

12. Une composition pour l'amplification d'un acide nucléique cible composée d'un lyophilisat unique possédant

   a) un taux efficace d'une activité ADN polymérase ARN-dépendante, d'une activité ADN polymérase ADN-dépendante, d'une activité ARNase H et d'une activité ARN polymérase ADN-dépendante parmi lesquelles l'activité ADN polymérase ARN-dépendante, l'activité ADN polymérase ADN-dépendante et l'activité ARNase H sont fournies par une ou plusieurs enzymes distinctes,
   b) un agent stabilisateur qui est composé soit d'un disaccharide non réducteur soit de polyvinylpyrrolidone,
   c) des désoxyribonucléotides-triphosphates t des ribonucléotides-triphosphates,
   d) des sels métalliques, et
   e) un agent réducteur, dans lequ l, lorsque ledit lyophilisat est reconstitué par addition d'un solvant aqueux,

la solution produite amplifiera une molécule d'ARN monocaténaire possédant une région de la séquence nucléotidique cible lors de l'addition de ladite molécule d'ARN et d'une ou plusieurs amorces oligonucléotidiques appropriées à ladite solution et de l'incubation de la solution à une température suffisante pour favoriser lesdites activités enzymatiques.

13. La composition selon la revendication 12 dans laquelle ladite ou lesdites plusieurs amorces appropriées sont contenues dans le lyophilisat.

14. La composition selon la revendication 13, comprenant en outre

f) un tampon, mais à la condition que ladite composition ne contienne pas un acide carboxylique.

15. La composition selon la revendication 12 ou 13 dans laquelle lesdites activité ADN polymérase ARN-dépendante, activité ADN polymérase ADN-dépendante et activité ARNase H sont fournies par une réverse transcriptase rétrovirale recombinante et ladite activité ARN polymérase ADN-dépendante est fournie par une ARN polymérase de bactériophage.

16. La composition selon la revendication 15 dans laquelle ladite réverse transcriptase est produite par le virus de la leucémie murine de Moloney.

17. La composition selon la revendication 15 dans laquelle ladite ARN polymérase est produite par le bactériophage T7.

18. La composition selon la revendication 15 dans laquelle ledit agent stabilisateur est de la polyvinylpyrrolidone.

19. La composition selon la revendication 14 dans laquelle ledit agent stabilisateur est du tréhalose.

20. Un kit pour l'amplification d'un acide nucléique cible comprenant une transcriptase réverse et une ARN polymérase combinées dans une composition lyophilisée unique conjointement avec un agent stabilisateur cryoprotecteur qui est composé soit d'un disaccharide non réducteur soit de polyvinylpyrrolidone, dans lequel lors de la réhydratation de ladite composition lyophilisée et de l'addition de l'acide nucléique cible en présence des amorces oligonucléotidiques, une partie ou la totalité de ladite acide nucléique cible sera amplifiée.

21. Le kit selon la revendication 20 dans lequel ladite composition lyophilisée comprend en outre des sels métalliques et des nucléotides-triphosphates.

22. Le kit selon la revendication 21 comprenant en outre au moins une amorce oligonucléotidique pour l'amplification.

23. Le kit selon l'une quelconque des revendications 20 à 22 dans lequel ledit agent stabilisateur est du tréhalose.

24. Le kit selon l'une quelconque des revendications 20 à 22 dans lequel ledit agent stabilisateur est de la polyvinylpyrrolidone.